

Cross-reactivities with *Cryptosporidium* spp. by chicken monoclonal antibodies that recognize avian *Eimeria* spp.

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Abstract

In a previous study, we have developed several chicken monoclonal antibodies (mAbs) against *Eimeria acervulina* (EA) in order to identify potential ligand molecules of *Eimeria*. One of these mAbs, 6D-12-G10, was found to recognize a conoid antigen of EA sporozoites and significantly inhibited the sporozoite invasions of host T lymphocytes in vitro. Furthermore, some of these chicken mAbs showed cross-reactivities with several different avian *Eimeria* spp. and the mAb 6D-12-G10 also demonstrated cross-reactivities with the tachyzoites of *Neospora caninum* and *Toxoplasma gondii*. *Cryptosporidium* spp. are coccidian parasites closely related to *Eimeria* spp., and especially *C. parvum* is an important cause of diarrhea in human and mammals. In the present study, to assess that the epitopes recognized by these chicken mAbs could exist on *Cryptosporidium* parasites, we examined the cross-reactivity of these mAbs with *Cryptosporidium* spp. using an indirect immunofluorescent assay (IFA) and Western blotting analyses. In IFA by chicken mAbs, the mAb 6D-12-G10 only showed a immunofluorescence staining at the apical end of sporozoites of *C. parvum* and *C. muris*, and merozoites of *C. parvum*. Western blotting analyses revealed that the mAb 6D-12-G10 reacted with the 48-kDa molecular weight band of *C. parvum* and *C. muris* oocyst antigens, 5D-11 reacted the 155 kDa of *C. muris*. Furthermore, these epitopes appeared to be periodate insensitive. These results indicate that the target

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antigen recognized by these chicken mAbs might have a shared epitope, which is present on the apical complex of apicomplexan parasites.

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1. Introduction

Cryptosporidium (Protozoa, Apicomplexa) causes cryptosporidiosis in man and economically important food animals throughout the world. Cryptosporidiosis is characterized by a self-limiting diarrhea in immunocompetent individuals but it can be chronic and life-threatening in immunocompromised patients (Colford et al., 1996; Pozio et al., 1997). Because specific immune responses can control cryptosporidiosis, passive immunization strategies have been investigated (Riggs, 1997; Crabb, 1998). However, to date, there are no approved vaccines, immunotherapy or effective pharmaceuticals for the prevention and treatment of cryptosporidiosis (Riggs, 1997; Jenkins et al., 1999; Perryman et al., 1999; Sagodira et al., 1999).

Cryptosporidium zoites including sporozoites and merozoites initiate infection by recognition, attaching to and invading host cells. Although the processes of invasion have not been well characterized, highly differentiated apical organelles of *Cryptosporidium* zoites are thought to play a key role in host-cell invasion (Bonnin et al., 1993). Therefore, new strategies for cryptosporidiosis control are being focused on understanding the nature of these organelles during the invasion process and elucidating whether some apical organelles could be candidate antigens.

Until now, monoclonal antibodies (mAbs) have been widely used for the identification of candidate antigens on *Cryptosporidium* zoites. Several mouse mAbs that recognize the apical regions or the surface molecules of invasive stages have been reported (Petersen et al., 1992; Riggs et al., 1997, 1999; Cevallos et al., 2000). However, in mouse immunization studies, the molecule was reported to be poorly immunogenic (Fayer et al., 1997), and optimal vaccine candidates that could completely prevent or terminate *Cryptosporidium* infection have not been identified.

In a previous study, we have developed several chicken monoclonal antibodies against *Eimeria*

acervulina (EA) to identify potential ligand molecules. Most of these chicken mAbs recognized the apical region of *Eimeria* invasive stages (Sasai et al., 1996; Constantinoiu et al., 2003). One of these mAbs, 6D-12-G10, was found to recognize a conoid antigen of EA sporozoites by immunoelectron microscopy and 21-kDa molecular weight of EA sporozoite antigen by Western blotting analyses, and significantly inhibited the sporozoite invasions of host T lymphocytes in vitro (Sasai et al., 1996). Thus, we reported that the antigen recognized by mAb 6D-12-G10 might be important for immunological therapy against EA infection. Furthermore, some of these chicken mAbs showed cross-reactivities with several different avian *Eimeria* spp. (Constantinoiu et al., 2003), and the mAb 6D-12-G10 also demonstrated a cross-reactivity with the tachyzoites of *Neospora caninum* and *Toxoplasma gondii* (Sasai et al., 1998). These results suggested that these chicken mAbs might recognize epitopes conserved in many species of cyst-forming coccidian. We hypothesized that epitopes recognized by these chicken mAbs might exist on *Cryptosporidium* zoites. To assess the hypothesis that *Cryptosporidium* antigens recognized by these chicken mAbs could be candidate vaccines for cryptosporidiosis, we tested cross-reactivities of these chicken mAbs with *Cryptosporidium* parasites.

2. Materials and methods

2.1. Parasites

C. parvum oocysts, strain HNJ-1, were originally obtained from the feces of a patient in Japan (Abe et al., 2002), and *C. muris* oocysts, strain RN 66, from mice in Japan (Iseki et al., 1989). These oocysts were passaged in severe combined immunodeficient (SCID) mice, purified by sugar flotation, and stored at 4 °C in a 2.5% potassium dichromate solution until 1 month before their use or freeze-dried for longer preservation.

2.2. Preparation of sporozoites

The *C. parvum* oocysts were washed three times with Hanks' balanced salt solution (HBSS) (Gibco BRL, Tokyo, Japan) by centrifugation at $1500 \times g$ for 10 min and were excysted by the method previously reported (Kato et al., 2001). Briefly, a million (10^6) oocysts of *C. parvum* were pretreated in HBSS containing 0.01N HCl at 37 °C for 1 h. After washing two times with HBSS, the sporozoites were excysted in HBSS containing 0.1% bovine bile solution (Wako, Osaka, Japan) for 10 min. The *C. muris* oocysts were washed three times with HBSS by centrifugation at $1500 \times g$ for 10 min and oocysts were resuspended in HBSS. Excystation of sporozoites was obtained by incubation of oocysts at 37 °C (Nina et al., 1992). In brief, 1 ml of HBSS containing 10^6 oocysts were incubated in flat-bottom 24-well tissue culture plates (Iwaki Grass, Tokyo, Japan) at 37 °C in an incubator with 5% CO₂ for 30 min. To conduct IFA, 50 µl mixture of excysted sporozoites and oocysts of *C. parvum* or *C. muris* was applied to clean siliconized slides (Dako Japan, Kyoto, Japan). Slide preparations were air-dried or fixed after drying using methanol or acetone, and stored at –80 °C until use.

2.3. Preparation of merozoites

For preparation of *C. parvum* merozoites, we used a modified method based on those previously reported (Slifko et al., 1997; Weir et al., 2001). Briefly, human colonic tumor (HCT-8) cells (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) were used for in vitro cultivation of *C. parvum*. HCT-8 cells were grown in a maintenance medium that consists of RPMI 1640 (Sigma Chemical Co., Tokyo, Japan) supplemented with 10% (v/v) fetal calf serum (FCS), 20 mM HEPES, 4 mM L-glutamine, 50 IU/ml penicillin, and 50 µg/ml streptomycin. Cells were maintained in 25 cm²-bottom plastic tissue culture flasks at 37 °C with 5% CO₂ and passaged every 3–4 days. For use in experiments, HCT-8 cells were trypsinized by Trypsin–EDTA solution (0.5% trypsin, 0.195% EDTA-2Na in PBS) (Gibco BRL), washed in the maintenance medium, resuspended at 4×10^5 /ml and cultured in perfusion chambers (Cover Well™, Grace Bio-Labs, Inc., Bend, Oregon) fixed on siliconized slide glasses (Matsunami, Osaka, Japan), containing 0.25 ml of maintenance medium per well for

72 h at 37 °C with 5% CO₂ to form a monolayer. To prepare merozoites, *C. parvum* oocysts were pretreated with 0.01N HCl for 30 min at 37 °C and washed with maintenance medium. HCT-8 cell monolayers were washed with maintenance medium before inoculation, and replaced with 0.25 ml of inoculation medium containing 0.1% bovine bile solution (Wako), which contains 2×10^5 ml^{–1} of oocysts. After oocysts inoculation, the HCT-8 cells were incubated at 37 °C with 5% CO₂ for 24 h. After incubation, the medium was aspirated and the perfusion chambers were removed from slide glasses. Then the infected monolayer on slide glasses was fixed in methanol or acetone for IFA, or 10% formaldehyde in PBS for confocal laser scanning microscopy (LSM510, Carl Zeiss Co., Tokyo, Japan), and stored at –80 °C until use.

2.4. Monoclonal antibodies

Six chicken monoclonal antibodies named 5D-11, 8D-2, 6D-12-G10, 8E-1, 8C-3 and HE-4 were produced as described (Sasai et al., 1996; Constantinoiu et al., 2003). The antibody isotypes were of the immunoglobulin G class and its reactivities were previously described (Sasai et al., 1996; Constantinoiu et al., 2003, 2004).

2.5. Indirect immunofluorescence assay

Prepared slides were incubated with 50 µl of culture supernatant from chicken hybridomas for 40 min at room temperature. The slides were washed three times with phosphate-buffered saline (PBS, pH 7.2) and incubated with 50 µl of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-chicken IgG (1:1000, Sigma) for 30 min. As a control, purified normal chicken IgG (1:1000, Sigma) was used. After staining, the slides were mounted with 50% glycerol in PBS for examinations using fluorescent (Nikon, Tokyo, Japan) and confocal laser scanning microscope (LSM510, Carl Zeiss Co.). All steps were performed at room temperature.

2.6. Western blot analysis

Freeze-dried *C. parvum* or *C. muris* oocysts were resuspended in PBS, sonicated in ice bath with ultrasonic processor (Taitec Co., Saitama, Japan) and

centrifuged at $600 \times g$ for 5 min. Supernatant was aliquoted and cryopreserved at -80°C until use. The concentration of crude antigen was determined by BCA protein assay kit (Pierce, Lockford, Illinois). The size of the target *C. parvum* or *C. muris* oocysts antigens that were recognized by the chicken mAbs was determined by Western blotting analyses. Briefly, 20–40 μg of *Cryptosporidium* oocysts antigens in sample buffer (125 mM Tris, 4% SDS, 20% glycerol, 10% β -mercaptoethanol, 0.0025% bromophenol blue) were heated at 95°C for 4 min. Molecular weight standard proteins (Bio-Rad, Tokyo, Japan) were also treated the same way as the parasite antigen. Sample was resolved on 4% stacking/12% resolving sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 200 V constant voltages (Laemmli, 1970) and the separated proteins were blotted to PVDF membrane (Immobilon Transfer Membranes, Millipore, Bedford, Massachusetts) using the Mini Trans-Blot[®] Electrophoretic Transfer Cell (Bio-Rad) in a buffer (50 mM Tris, 192 mM glycine, 20% methanol) for 1 h at 100 V constant voltages. After blotting, the membrane was air-dried. Individual lanes were stained for 1 h at room temperature with each of the chicken mAbs or a chicken-purified IgG as a control and washed three times with PBS containing 0.05% Tween 20 for 5 min. Bound antibodies were detected by staining with horseradish peroxidase-labeled rabbit anti-chicken IgG F(ab)₂ fragment (1:1000, Cortex Biochem., California, USA) for 1 h and developed with Konica Immunostaining HRP-1000 (Konica, Tokyo, Japan). Molecular weights were estimated using molecular weight standard proteins after staining with 0.2% Coomassie Brilliant Blue R-250 (Wako).

2.7. Determination of carbohydrate epitopes

To determine whether the epitopes contain carbohydrate, we used the procedure developed by Woodward et al. (1985) for antigens on Western blots. Briefly, each PVDF strip transferred electrophoretically after SDS-PAGE was incubated with 10 or 50 mM sodium *m*-periodate in 50 mM sodium acetate buffer (pH 4.5) in a dark environment at room temperature for 1 h, and was then exposed to 50 mM sodium borohydride in PBS for 30 min. Controls were incubated in the same buffer in the absence of

periodate. Following three washes with PBS, the PVDF strips were probed with mAbs as described above.

3. Results

3.1. Indirect immunofluorescence analysis

In IFA with six chicken mAbs, the mAb 6D-12-G10 only reacted at the apical portion of the methanol, acetone fixed and non-fixed sporozoites of *C. parvum* and *C. muris* (Figs. 1 and 2). The mAb 6D-12-G10 did not react with the oocyst wall. Other chicken mAbs and purified normal chicken IgG did not show any binding on *C. parvum* and *C. muris* sporozoites or oocysts (data not shown).

In HCT-8 cells that were infected with *C. parvum*, asexual stages were observed at 24 h after inoculation. In IFA of cultured *C. parvum* zoites fixed by methanol or acetone, only the mAb 6D-12-G10 showed reactivities. Fig. 3A and B shows the meronts during the early stage of development at 24 h after the inoculation into host cells. The mAb 6D-12-G10 showed reactivity with zoites, which were present in the outermost layer of the immature meront (Fig. 3B). Fig. 3C–F shows the mature meront stage including merozoites, which were fixed using methanol or acetone. The positive staining was observed at the apical region of the merozoites within mature meronts. Other chicken mAbs and purified normal chicken IgG did not show any reactions (data not shown). By confocal laser scanning microscopy, we could confirm cultivated type I meront because more than four positive merozoites in one meront were seen (Fig. 3G). After 24 h incubation, other stages of *C. parvum* were not observed.

3.2. SDS-PAGE and immunoblot analysis of *C. parvum* and *C. muris* oocysts antigens with chicken mAbs

By Western blotting analyses of the solubilized *Cryptosporidium* oocysts antigens with chicken mAbs, only the mAb 6D-12-G10 recognized predominantly the 48-kDa antigens of *C. parvum* and *C. muris* (Fig. 4). The positive band of 155 kDa reactive with the mAb 5D-11 was only observed with *C. muris*.

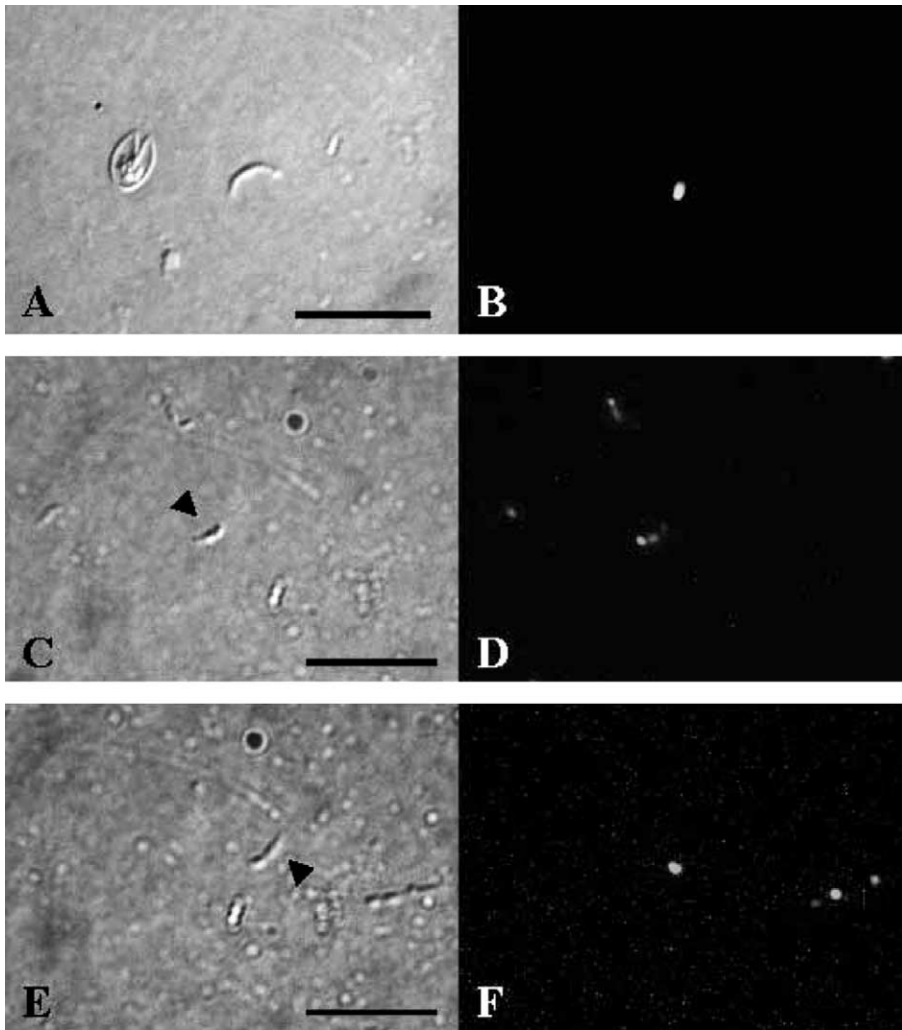


Fig. 1. Indirect immunofluorescence staining of *C. parvum* sporozoites with chicken mAb 6D-12-G10. Photomicrographs from bright field by interference contrast microscopy (A, C, E) and immunofluorescence microscopy (B, D, F) are shown. *C. parvum* sporozoites were fixed by methanol (A, B), acetone (C, D) or non-fixed (E, F). Arrowheads indicate sporozoites of *C. parvum*. Bar = 10 μ m.

None of the other mAbs and chicken-purified IgG showed any reactions. The left lane is molecular weight standard.

3.3. Periodate sensitivity

The reactivities of the mAbs 6D-12 and 5D11 to *C. parvum* and *C. muris* antigens treated with 10 or 50 mM sodium *m*-periodate are shown in Fig. 5. By periodate treatment, the mAbs 6D-12 and 5D11 still recognized the 48-kDa antigens of *C. parvum* and *C.*

muris, and the 155-kDa of *C. muris*, respectively. The left lane is molecular weight standard.

4. Discussion

In the present study, we showed the cross-reactivities of chicken mAbs with *Cryptosporidium* parasites. By IFA of six chicken mAbs, the mAb 6D-12-G10 only reacted with *C. parvum* and *C. muris* sporozoites, and *C. parvum* merozoites. To date, few

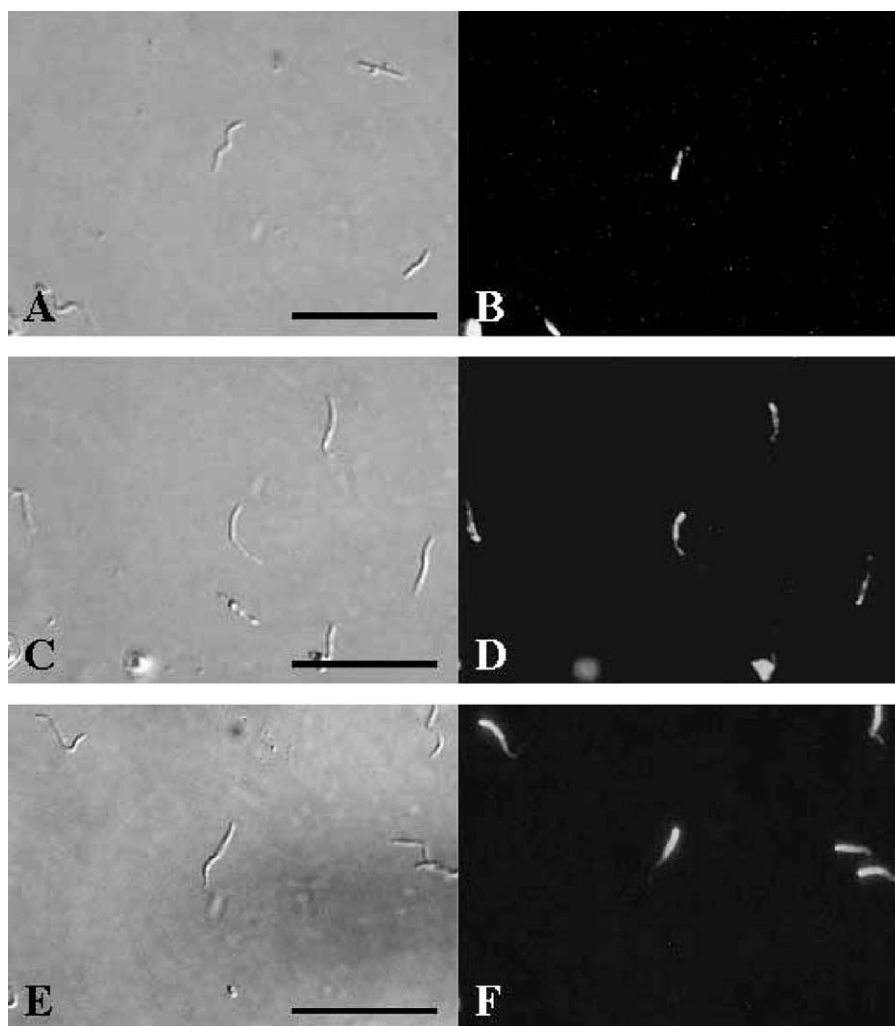


Fig. 2. Indirect immunofluorescence staining of *C. muris* sporozoites with the chicken mAb 6D-12-G10. Photomicrographs from bright field by interference contrast microscopy (A, C, E) and immunofluorescence microscopy (B, D, F) are shown. *C. parvum* sporozoites were fixed by methanol (A, B), acetone (C, D) or non-fixed (E, F). Bar = 10 μ m.

reports of mAbs which showed cross-reactivities with zoites of *Cryptosporidium* and other apicomplex parasites were available. Our results suggested that the antigen recognized by mAb 6D-12-G10 could highly share among the apicomplexa parasites, including *Eimeria*, *Toxoplasma*, *Neospora*, and *Cryptosporidium*.

In IFA, the positive reactions localized only to the anterior region of invasive stages. The mAb 6D-12-G10 recognized external antigen of zoites because the mAb 6D-12-G10 reacted with non-fixed as well as

methanol- or acetone-fixed sporozoites. Although we have reported that the mAb 6D-12-G10 recognized the conoid antigens of EA sporozoites (Sasai et al., 1996), we could not determine the precise organelles on *Cryptosporidium* sporozoites and merozoites. At the anterior end of invasive forms in phylum Apicomplexa, a unique complex of organelles which are made of rhoptries, dense granules and micronemes is located (Sam-Yellowe, 1996), and the conoid, polar rings and subpellicular microtubules are believed to provide a cytoskeletal framework during host cell invasion

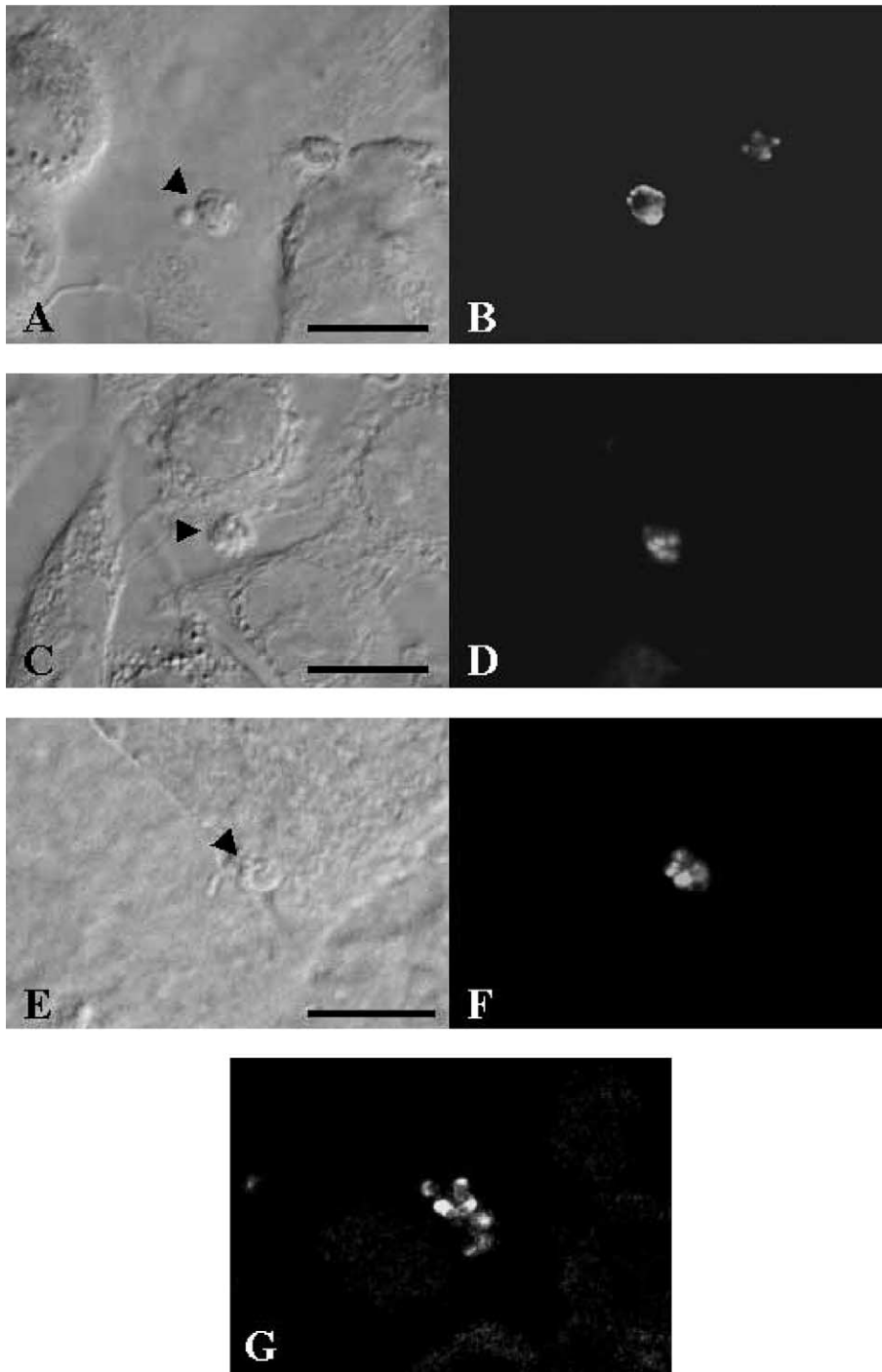


Fig. 3. Indirect immunofluorescence staining of in vitro cultivated *C. parvum* zoites with the chicken mAb 6D-12-G10. Photomicrographs from bright field by interference contrast microscopy (A, C, E), immunofluorescence microscopy (B, D, F) and confocal laser scanning microscopy (G) are shown. At 24 h after inoculation, cultivated *C. parvum* zoites were fixed by methanol (A–D), acetone (E, F) or formaldehyde (G). *C. parvum* immature meront on early stage (A, B) and *C. parvum* mature meront, merozoites (C–G) were shown. Bar = 10 μ m.

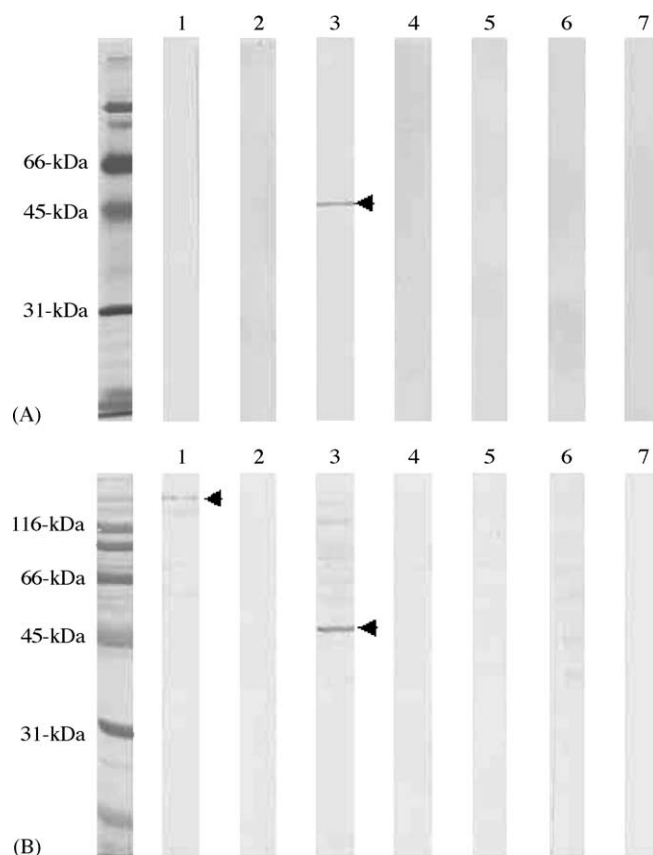


Fig. 4. Western blot analysis of *C. parvum* (A) and *C. muris* (B) antigens with six different chicken mAbs. Lanes: 1, mAb 5D-11; 2, mAb 8D-2; 3, 6D-12-G10; 4, 8E-1; 5, 8C-3; 6, HE-4; 7, normal chicken IgG. The left lane is molecular weight standard. Arrowheads indicate positive bands.

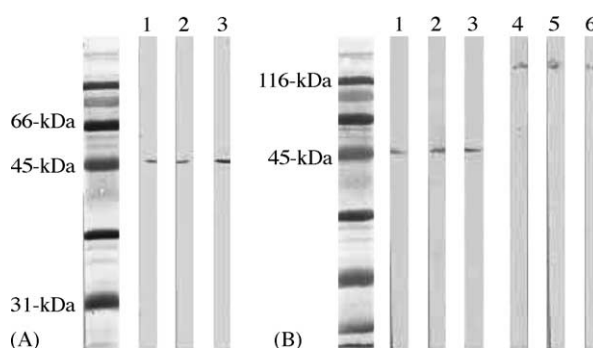


Fig. 5. Western blot analysis of *C. parvum* (A) and *C. muris* (B) after *m*-periodate treatment. Lanes 1 and 4 were incubated in buffer without *m*-periodate, lanes 2 and 5 were with 10 mM *m*-periodate, lanes 3 and 6 were with 50 mM *m*-periodate. 1–3 lanes were probed with chicken mAb 6D-12-G10 and 4–6 lanes were 5D-11, respectively. The left lane is molecular weight standard.

(Lindsay et al., 1991, 1993; Hu et al., 2002). Thus, the mAb 6D-12-G10 might recognize one or more of these organelles on *Cryptosporidium* zoites. However, there is little information about the ultrastructural details of *Cryptosporidium* sporozoites and merozoites (Uni et al., 1987; Lumb et al., 1988), or the role of these organelles in host–parasite interaction.

The mAb 6D-12-G10 showed cross-reactivity with the in vitro cultivated zoites of *C. parvum*. At 24 h after infection to host cells with *Cryptosporidium* oocysts, meronts including merozoites were localized on the surface of host cell (Fig. 3A, C and E). In IFA, positive reactions were seen in immature meronts and the apical region of merozoites within type I meronts. Thus, these results suggested that the apical antigen recognized by the mAb 6D-12-G10 was expressed on the zoites during the early stage of merogony, and was conserved among the zoites of the motile stages of *Cryptosporidium* parasites.

On Western blotting analyses, the mAb 6D-12-G10 recognized a 48-kDa band associated with *C. parvum* and *C. muris* oocysts antigens, and the mAb 5D-11 recognized 155-kDa of *C. muris*. In a previous study, the molecular weight of EA antigen recognized by the mAb 6D-12-G10 was 21 kDa. These results showed that the molecular mass including epitope recognized by mAb 6D-12-G10 was different between *Cryptosporidium* and *Eimeria*. Although the mAb 5D-11 showed no reactions with *Cryptosporidium* zoites or oocysts in IFA, the mAb 5D-11 recognized 155 kDa of only *C. muris* antigen. Until now, there are no reports about 155-kDa antigen on *C. muris*. Although the immunolocalization of *C. muris* recognized by the mAb 5D-11 remains unknown, the 5D-11 might recognize the internal oocyst or sporozoite antigen of *C. muris*, or only solubilized antigens of *C. muris*.

There are very few reports of mouse mAbs, which specifically recognize the apical complex of *Cryptosporidium*, compared to the number of mouse mAbs which recognize surface antigens of invasive stages or oocyst wall. Some papers have reported the development of mAbs, which recognized the apical organelles of *C. parvum* zoites, e.g., micronemes (Bonnin et al., 1991, 2001; Robert et al., 1994), and dense granules (Bonnin et al., 1995), but there are no papers about other organelles. On Western blotting analyses, these mAbs reacted with the high-molecular weight antigens of more than 100 kDa, which are much higher

than that of the antigen recognized by mAb 6D-12-G10. Furthermore, most epitopes recognized by these mAbs were reported to be periodate sensitive (glycosylated). Of these mAbs previously reported, the epitope recognized only by the mAb HAD was periodate insensitive (Bonnin et al., 1991). The mAb HAD was thought to recognize *Cryptosporidium* microneme antigens. However, this mAb recognized 63–210 kDa antigens. Thus, the 48-kDa antigen recognized by the mAb 6D-12-G10 would be a new one.

In the present study, we first examined reactivities with *Cryptosporidium* parasites by chicken mAbs. Immune systems of chickens are different from those of mammals in mechanisms of B cell repertoire formation (Reynaud et al., 1994), and chicken IgGs are considered to be useful for immunochemical research and clinical application (Nishinaka et al., 1996). By comparing with *Cryptosporidium* antigens recognized by mouse mAbs, the antigens recognized by chicken mAbs in the present study were found to be novel. Furthermore, the epitope recognized by the mAb 6D-12-G10 was found to be highly conserved among apicomplexan, parasites including *Eimeria*, *Toxoplasma*, *Neospora* and *Cryptosporidium*. Although further studies are needed to characterize these antigens of *Cryptosporidium*, these chicken mAbs, in particular 6D-12-G10, may have use as analytical tools for research on cryptosporidiosis.

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